Available online at www.sciencedirect.com
SCIENCE DIRECT
Developmental Biology 287 (2005) 346 – 360
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Interactions between muscle fibers and segment boundaries in zebrafish

Clarissa A. Henry [a,1], Ian M. McNulty [b], Wendy A. Durst [a], Sarah E. Munchel [a], Sharon L. Amacher [a,*]

[a] Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200, USA
[b] Department of Biological Sciences, University of Maine, Orono, ME 04469-2988, USA

Received for publication 21 March 2005, revised 28 August 2005, accepted 30 August 2005
Available online 12 October 2005

Abstract

The most obvious segmental structures in the vertebrate embryo are somites: transient structures that give rise to vertebrae and much of the musculature. In zebrafish, most somitic cells give rise to long muscle fibers that are anchored to intersegmental boundaries. Therefore, this boundary is analogous to the mammalian tendon in that it transduces muscle-generated force to the skeletal system. We have investigated interactions between somite boundaries and muscle fibers. We define three stages of segment boundary formation. The first stage is the formation of the initial epithelial somite boundary. The second "transition" stage involves both the elongation of initially round muscle precursor cells and somite boundary maturation. The third stage is myotome boundary formation, where the boundary becomes rich in extracellular matrix and all muscle precursor cells have elongated to form long muscle fibers. It is known that formation of the initial epithelial somite boundary requires Notch signaling; vertebrate Notch pathway mutants show severe defects in somitogenesis. However, many zebrafish Notch pathway mutants are homozygous viable suggesting that segmentation of their larval and adult body plans at least partially recovers. We show that epithelial somite boundary formation and slow-twitch muscle morphogenesis are initially disrupted in *after eight* (*aei*) mutant embryos (which lack function of the Notch ligand, DeltaD); however, myotome boundaries form later ("recover") in a Hedgehog-dependent fashion. Inhibition of Hedgehog-induced slow muscle induction in *aei/deltaD* and *deadly seven* (*des*)*/notch1a* mutant embryos suggests that slow muscle is necessary for myotome boundary recovery in the absence of initial epithelial somite boundary formation. Because we have previously demonstrated that slow muscle migration triggers fast muscle cell elongation in zebrafish, we hypothesize that migrating slow muscle facilitates myotome boundary formation in *aei/deltaD* mutant embryos by patterning coordinated fast muscle cell elongation. In addition, we utilized genetic mosaic analysis to show that somite boundaries also function to limit the extent to which fast muscle cells can elongate. Combined, our results indicate that multiple interactions between somite boundaries and muscle fibers mediate zebrafish segmentation.

Keywords: Somite; Muscle; Notch; *her1*; *her7*; *b567*; *after eight/deltaD*; Hedgehog; Myoseptum; Myotome

Introduction

Segmentation of the vertebrate body plan is a crucial aspect of normal development. In vertebrates, mesodermal segmentation involves partitioning of unsegmented presomitic mesoderm into discrete structures called somites. Somites give rise to skeletal structures such as ribs and vertebrae as well as skeletal muscle. In zebrafish as well as chick, short range cell rearrangements underlie the morphogenesis of somite boundaries (Henry et al., 2000; Kulesa and Fraser, 2002). Notch signaling is critical for somite boundary formation in zebrafish, chicken, *Xenopus*, and mouse (Dale et al., 2003; Pourquie, 2003a,b). For example, several zebrafish mutations that affect somite boundary formation (*after eight/deltaD, deadly seven/notch1a, mind bomb*, and *beamter/deltaC*) are known to disrupt Notch pathway components (Holley et al., 2000, 2002; Itoh et al., 2003; Jiang et al., 1998). In addition, we and others have demonstrated that zebrafish *hairy/Enhancer of split*-related genes, *her1* and *her7*, which encode downstream targets of the Notch pathway, are required for normal somite formation (Gajewski et al., 2003; Henry et al., 2002; Holley et al., 2002; Oates and Ho, 2002). Interestingly, some zebrafish

* Corresponding author.
E-mail addresses: Clarissa.Henry@umit.maine.edu (C.A. Henry), amacher@uclink4.berkeley.edu (S.L. Amacher).
[1] Current address: Department of Biological Sciences, University of Maine, Orono, ME 04469-2988, USA.

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.08.049

homozygous Notch pathway mutants, *after eight/deltaD*, *deadly seven/notch1a*, and *beamter/deltaC*, are viable and survive to adulthood (van Eeden et al., 1996) indicating that even though the initial process of somite formation is disrupted, segmentation at least partially recovers. This partial recovery of segmentation would pattern the imperfect formation of segmental elements such as vertebrae and skeletal muscle in mutant embryos. We investigated how segmentation recovers in Notch pathway mutants.

Typically, two types of cells are found in developing somites: epithelial border cells and inner mesenchymal cells. As a somite matures, presumptive muscle cells elongate either actively or through fusion to form long muscle fibers (Cortes et al., 2003; Denetclaw et al., 1997, 2001; Devoto et al., 1996; Henry and Amacher, 2004; Kahane et al., 1998; Kalcheim et al., 1999; Kielbowna, 1981; Neff et al., 1989; Venters et al., 1999). In zebrafish, muscle fibers attach to the extracellular matrix at both the anterior and posterior segment boundaries, forming the body wall musculature. The somite boundary undergoes a significant morphogenesis, becoming rich in extracellular matrix and adhesion complex components. Components of both the dystroglycan complex and focal adhesions concentrate at the somite boundary (Bassett et al., 2003; Crawford et al., 2003; Dubois et al., 2002; Henry et al., 2001; Kudo et al., 2004; Parsons et al., 2002), which is now called a myotome boundary. Thus, boundary formation involves multiple steps that we define in this work as follows. Stage 1 is the formation of the initial epithelial somite boundary. Stage 2 is a transition stage, where muscle cells are elongating to generate the myotome and the somite boundary is becoming rich in extracellular matrix. Stage 3 is the formation of the myotome boundary, which is exceedingly rich in matrix and adhesion components; during this stage, muscle precursor cells have fully elongated to form muscle fibers. Although a great deal is known about the signaling pathways necessary for initial epithelial somite boundary formation, less is known about myotome boundary formation.

One process involved in myotome boundary formation is the elongation of muscle precursor cells to generate the myotome: a group of specified, elongated, muscle fibers. We have recently shown that fast-twitch muscle cell elongation is triggered by slow-twitch muscle cell migration (Henry and Amacher, 2004). Specification/commitment of the slow-twitch muscle lineage is dependent upon Hedgehog signaling (Barresi et al., 2000; Blagden et al., 1997; Coutelle et al., 2001; Currie and Ingham, 1996, 1998; Du et al., 1997; Hirsinger et al., 2004; Lewis et al., 1999; Roy et al., 2001). High levels of Hedgehog signaling induce Engrailed-expressing muscle pioneers, a subset of slow-twitch muscle cells located at the horizonal myoseptum, and a small subset of fast fibers, the Engrailed-expressing medial fast fibers. Slightly lower levels of Hedgehog signaling induce superficial slow fibers, which migrate from their initial position adjacent to the notochord laterally through the paraxial mesoderm and become the most superficial muscle fibers (Devoto et al., 1996; Wolff et al., 2003). This migration is dependent upon reciprocal waves of N- and M-cadherin expression (Cortes et al., 2003). Thus, slow muscle specification requires Hedgehog signaling, and slow muscle morphogenesis requires dynamic modulation of N- and M-cadherin expression. Furthermore, slow muscle fibers are critical not only for slow-twitch muscle development, but also for normal fast-twitch muscle fiber elongation (Henry and Amacher, 2004).

In this study, we examine the relationship between muscle cell elongation and myotome boundary formation. Initial epithelial somite boundaries do not form in Notch pathway mutant embryos. However, slow muscle cells do elongate. The elongation and morphogenesis of slow muscle cells in the absence of initial somite boundary formation in *aei/deltaD* mutant embryos are disorganized. This suggests that initial epithelial somite boundary formation may help establish the normal pattern of slow muscle cell elongation. We show that later in development, by 24 h post-fertilization, irregular but robust myotome boundaries do form in *aei/deltaD* mutant embryos. We predicted that slow muscle migration may pattern not only fast muscle cell elongation, but also myotome boundary recovery in Notch pathway mutants, as has been proposed for another somite boundary mutant (van Eeden et al., 1998). In support of this hypothesis, we find that Hedgehog signaling is necessary for myotome boundary recovery in *aei/deltaD* and *des/notch1a* mutant embryos. Finally, we show using mosaic analysis that one function of somite and/or myotome boundaries is to limit the extent to which muscle cells can elongate in vivo. Therefore, our results provide insights into the integrated mechanisms of muscle cell elongation and somite boundary maturation.

Materials and methods

Zebrafish mutant alleles, stocks, and husbandry

Zebrafish embryos were obtained from natural spawnings of adult fish kept at 28.5°C on a 14 h light/10 h dark cycle and were staged according to Kimmel et al. (1995). The alleles of *mind bomb* (*mib*^{ta52b}), *after eight/deltaD* (*aei*^{tr233}), and *deadly seven/notch1a* (*des*^{b638}) used have been previously described (Gray et al., 2001; Holley et al., 2000, 2002; Itoh et al., 2003; van Eeden et al., 1996). An allele of *beamter* (*bea*^{b663}) was isolated in an in situ hybridization screen (see Henry et al., 2002 for details of the screen), and fails to complement the original allele (van Eeden et al., 1996).

In situ hybridization and immunocytochemistry

Whole mount in situ hybridization was performed as previously described (Jowett, 1999). Alexa Fluor 546 phalloidin was obtained from Molecular Probes. β-catenin (Sigma, C7207) and pY397 FAK (Biosource) staining was also performed as previously described (Henry et al., 2001; Topczewska et al., 2001). F59 antibody recognizes slow muscle myosin and was generously provided by Frank Stockdale. F59 staining was performed as previously described (Devoto et al., 1996). To double stain embryos with phalloidin and F59, embryos were fixed in 4% PFA, rinsed, incubated in 2% triton for 1.5 h, then incubated in 1:20 phalloidin 1 h, rinsed overnight in PBT, and then processed per established protocols. Laminin and Fibronectin antibodies were obtained from Neomarkers and staining was performed as previously described (Crawford et al., 2003).

Confocal microscopy

Confocal images were taken on a Zeiss Confocal at the Molecular Imaging Center at UC Berkeley and images were processed using both Zeiss LSM Image Analyzer and Adobe Photoshop. For all mutants discussed, at least 6

mutant embryos were examined. 3D reconstruction was performed with Zeiss Image Analyzer Software. Frequently, black and white were inverted in Adobe Photoshop to facilitate visualization.

Cyclopamine treatment

Embryos were treated with cyclopamine (Toronto Research Chemicals) to disrupt Hedgehog signaling as previously described (Barresi et al., 2001). In brief, embryos were incubated in 100 μM cyclopamine (with 1% EtOH) beginning at shield stage (6 hpf) until fixation. This concentration of cyclopamine has been recently shown to reduce *ptc1* transcripts by at least 50%, result in a complete loss of Engrailed expression and muscle pioneers, and have a significant reduction in the number of superficial slow muscle fibers (Wolff et al., 2003). As cyclopamine does precipitate when placed into embryo rearing medium, it is likely that there is batch-to-batch variability. In our batch of cyclopamine, 100 μM cyclopamine completely eliminated superficial slow muscle fibers at 24 h (data not shown). Controls were treated with 1% EtOH. In each experiment, cyclopamine-treated embryos had u-shaped somites and were partially or fully cyclopic. Furthermore, either *myoD* expression was examined in 5 control embryos and 5 cyclopamine-treated embryos from each experiment to verify absence of *myoD* expression in adaxial slow muscle precursor cells upon cyclopamine treatment, or embryos were stained with F59 to confirm loss of slow muscle fibers.

Mosaic analysis

Isochronic transplantations were performed at the blastula stage as previously described (Amacher and Kimmel, 1998). Donor embryos from an intercross of *b567* heterozygous embryos were uniformly labeled with lineage tracer dye (4% tetramethyl rhodamine-dextran). *b567* is a deficiency that lacks *her1* and *her7* and has defects in somite boundary formation (Henry et al., 2002). Cells were removed from donor embryos and placed into the blastoderm margin of unlabeled host embryos. Because transplantations were performed before embryos could be morphologically identified, donors were kept alive and scored as *b567* or wild-type. Hosts were fixed and processed for β-catenin and Fak expression as described above.

Results

Muscle fiber elongation proceeds medially to laterally in Notch pathway mutant embryos

Many zebrafish Notch pathway mutants that disrupt initial somite boundary formation have been described (Henry et al., 2002; Holley et al., 2000, 2002; Itoh et al., 2003). However, many of these mutants are homozygous viable indicating that segmentation in these embryos recovers through time. We hypothesized that muscle cell elongation may contribute to segmental recovery and analyzed the pattern of muscle cell elongation in Notch pathway mutant embryos (Fig. 1). Fast muscle cell elongation proceeds in a medial to lateral direction during muscle development (Henry and Amacher, 2004; Figs. 1A, A′). In *des/notch1a* and *aei/deltaD* homozygous mutant embryos, the first 7–9 somites form but somite formation is disrupted posteriorly (Holley et al., 2000, 2002; van Eeden et al., 1996). Here, we find that even in the absence of initial epithelial somite boundary formation, muscle cell elongation in mutant embryos proceeds in a medial to lateral fashion throughout the anterior–posterior axis in Notch pathway mutant embryos (Figs. 1B, B′, C, C′; compare long muscle cells in medial panels [on the left] to short muscle cell precursors in lateral panels [on the right], and data not shown). Interestingly, divisions that resemble somite boundaries tend to appear between these stacks of elongated muscle cells (arrowheads, Figs. 1B, C). Weak hints of somite boundaries are also observed laterally (arrowheads, Figs. 1B′, C′, D′, E′), but these boundaries do not persist throughout the dorso-ventral and medio-lateral extents of the paraxial mesoderm.

The *mind bomb* gene encodes an E3 ubiquitin ligase which ubiquitinylates zebrafish *deltaD* (Itoh et al., 2003). The somitic phenotype of *mind bomb* mutants is very similar to *aei/deltaD* and *des/notch1a* mutant embryos, except that somite defects initiate at slightly more posterior positions, around somite 10–11 (Fig. 1D′; data not shown). However, muscle cell elongation does proceed in a medial to lateral progression (Figs. 1D, D′). An additional Notch pathway mutation, *beamter*, disrupts *deltaC* (S.A. Holley and Y.-J. Jiang, personal communication). We analyzed muscle cell elongation in *bea/deltaC* mutant embryos and find that it also proceeds in a medial to lateral progression (Figs. 1E, E′).

Although muscle cell elongation proceeds in a medial to lateral fashion in all Notch pathway mutant embryos examined, muscle cell elongation is less organized than in wild-type embryos. Specifically, although muscle cells tend to elongate in dorsal–ventral stacks (arrow, Fig. 1B), these stacks can be of varying length and may not persist throughout the entire dorso-ventral or anterior–posterior extent of the paraxial mesoderm (see Figs. 1C, D, E, arrows). However, whereas there are only a few hints of cellular organization laterally (Figs. 1B′–E′), these stacks of elongated cells demonstrate cellular organization that could potentially underlie segmental recovery in these embryos.

Three stages of segment boundary formation

The medial to lateral progression of muscle cell elongation is preserved in Notch pathway mutant embryos and demarcations that resemble somite boundaries do form in between elongating muscle cells (Fig. 1). To address the issue of somite boundary recovery in Notch pathway mutants, we investigated the progression of somite boundary formation through time in both wild-type and *aei/deltaD* mutant embryos.

We find that there are three basic stages to segment boundary formation in wild-type embryos. The first stage is the formation of the initial epithelial somite boundary (Figs. 2A, A′). This initial somite boundary is flanked by epithelial border cells that surround an inner mass of mesenchymal cells (Henry et al., 2000). Focal adhesion components show a subtle localization to this initial somite boundary (Crawford et al., 2003; Henry et al., 2001). The second stage of segment boundary formation is a transitional stage from initial epithelial somite boundary formation to myotome boundary formation (Figs. 2C, C′, D, D′). During this stage, both the somite boundary and somitic cells are undergoing morphogenesis. Muscle precursor cells are elongating into muscle fibers in a medial to lateral progression (Fig. 2C′, arrowhead denotes elongating cells). Also, during this time, focal adhesion components like phosphorylated Focal Adhesion Kinase show a more robust accumulation at the somite boundary (Figs. 2C,

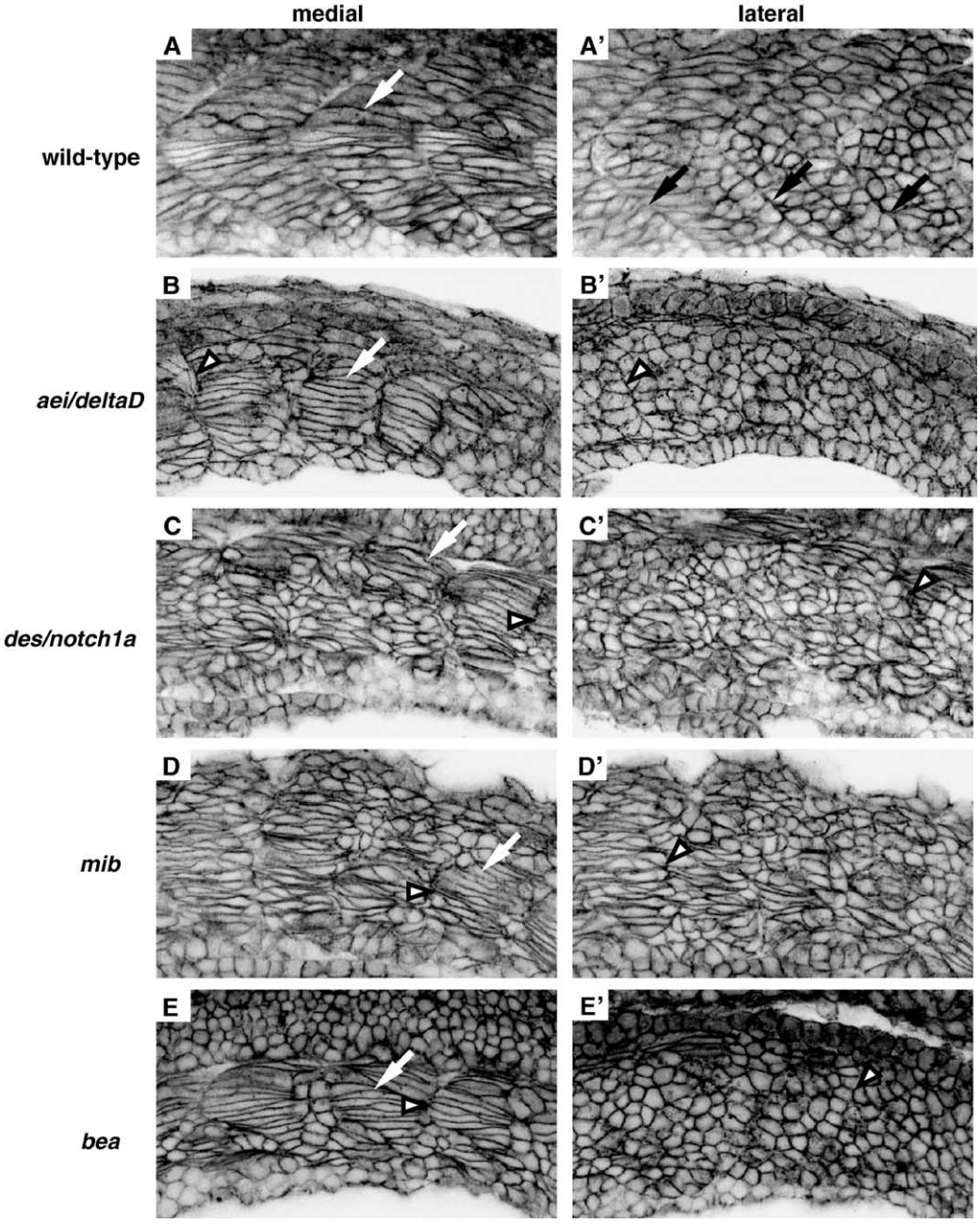


Fig. 1. Muscle fiber elongation initiates and proceeds medially to laterally in known and presumptive Notch pathway mutant embryos lacking normal posterior epithelial somite boundaries. Confocal micrographs (side views, anterior left) of 17–18 hpf embryos stained with β-catenin; contrast has been inverted so cells are outlined in black. Somite 10, or the approximate location of somite 10, is on the left. Left panels are medial (4–8 μm lateral to the notochord); right panels, marked with a prime, are more lateral (12–16 μm lateral to the notochord). In wild-type embryos, muscle cell elongation initiates medially (A, white arrow denotes long muscle cell), and somite boundaries persist throughout the paraxial mesoderm (A′, black arrows). Medially in *aei/deltaD* mutant embryos, muscle cells elongate in dorsal–ventral stacks (B, white arrow), resulting in the formation of boundaries (arrowhead) between these stacks. Thus, muscle cell elongation initiates even though initial somite boundary formation is severely disrupted (B′, arrowhead denotes a weak somite boundary). In *des/notch1a* (C), *mib* (D), and *bea* (E) mutant embryos, muscle cell elongation also initiates (white arrows) and is organized, resulting in boundaries between stacks of elongated muscle cells (arrowheads). Again, initial epithelial somite boundary formation is clearly disrupted (C′, D′, E′, arrowheads denote weak somite boundaries that do not persist throughout the dorsal–ventral and medial–lateral dimensions of the paraxial mesoderm).

D). Finally, the third stage of segment boundary formation is myotome boundary formation. At this time, all muscle precursors have elongated to generate the myotome (Figs. 2G, G′, H, H′). The myotome boundary at this point is exceedingly rich in extracellular matrix and focal adhesion components (Figs. 2G, G′, H, H′; Crawford et al., 2003; Henry et al., 2001), as well as components of the dystroglycan complex (Bassett et al., 2003; Guyon et al., 2003; Parsons et al., 2002).

To characterize boundary recovery in Notch pathway mutant embryos, we used the above methods to carefully examine the segmentation process in *aei/deltaD* mutant embryos. As

previously mentioned, somite boundary formation (Stage 1) is disrupted in Notch pathway mutant embryos, and strong or robust somite boundaries that persist throughout the dorsal–ventral and medial–lateral extent of the paraxial mesoderm do not form posteriorly (Fig. 1). However, weak somite boundaries do form in *aei/deltaD* mutant embryos, and Fibronectin localizes to those boundaries as in wild-type embryos (Figs. 2B, B′). This indicates that although somite formation is spatially disrupted and disorganized in *aei/deltaD* mutant embryos, weak boundaries appear to form by epithelialization of border cells and matrix deposition as in wild-type embryos.

During the transitional Stage 2, muscle cells begin to elongate in *aei/deltaD* mutant embryos (Figs. 1, 2E), and there are some weak somite boundaries that express phosphorylated Focal Adhesion Kinase (Fak) (Figs. 2E, F). However, at this stage, robust boundaries that persist through much of the medial to lateral and dorsal to ventral extent of the paraxial mesoderm are not observed in the posterior of *aei/deltaD* mutant embryos.

Robust, but irregular, boundaries do form by Stage 3 (myotome boundary formation) in *aei/deltaD* mutant embryos. The boundaries extend throughout most of the dorsal–ventral and medial–lateral extents of the paraxial mesoderm and show robust localization of phosphorylated Fak as in wild-type embryos (Figs. 2I, I′ J, J′). Therefore, our data show that robust segment boundaries recover as early as 24 h post-fertilization (hpf) in *aei/deltaD* mutant embryos.

The formation of irregular somite boundaries by 24 hpf of development is not unique to *aei/deltaD* mutant embryos. There is robust, albeit imperfect, recovery of myotome boundary development in other Notch pathway mutant embryos (Fig. 3).

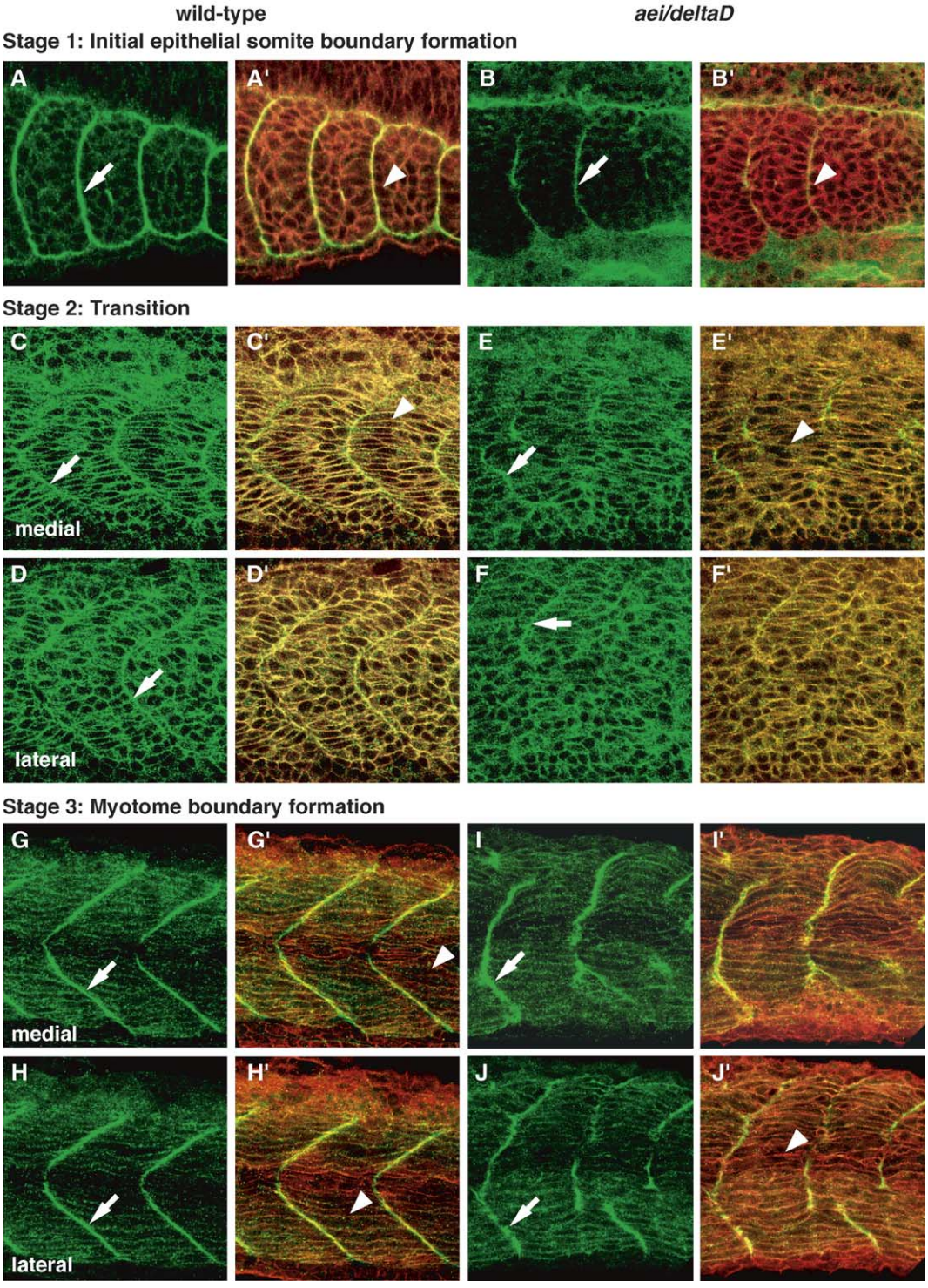
Slow-twitch muscle morphogenesis is initially disrupted but significantly recovers in aei/deltaD mutant embryos

One early marker of slow muscle specification is the expression of the myogenic regulatory factor *myoD* in slow muscle progenitor cells (Devoto et al., 1996; Weinberg et al., 1996). The normal initiation of *myoD* expression in Notch pathway mutant embryos (van Eeden et al., 1996) suggests that slow muscle specification is normal in Notch pathway mutant embryos. Because muscle fibers normally elongate between somite boundaries, we hypothesized that there may be morphogenetic defects in slow muscle in *aei/deltaD* mutant embryos due to the lack of initial epithelial somite boundaries. To test this hypothesis, we analyzed slow muscle morphogenesis in 20 hpf embryos, a time when superficial slow muscle fibers have begun their dramatic medial to lateral migration. In wild-type embryos, slow muscle cells elongate between two somite boundaries and are thus of uniform length (Fig. 4A, slow muscle myosin is in blue and rhodamine phalloidin stains all cells in red). Furthermore, there is a three-dimensionality to slow muscle cell distribution with muscle pioneers medial (Fig. 4A″, black *) and migrating superficial slow fibers more lateral (Fig. 4A″, blue *; Devoto et al., 1996). In *aei/deltaD* mutants, however, slow muscle fibers are of varying length (Fig. 4B), and there are somitic regions where slow fibers are missing. Furthermore, the normal three-dimensional architecture to the slow muscle is abolished and slow muscle is scattered throughout most of the medial–lateral extent of the somite (Fig. 4B″). These data indicate that the initial morphogenesis of slow-twitch muscle fibers is disrupted in *aei/deltaD* mutant embryos. However, by 26 hpf, when slow muscle fibers have significantly matured and fully migrated to the lateral extent of the wild-type somite (Figs. 4C, C′, C″), there is significant recovery of slow-twitch muscle fiber morphogenesis in *aei/deltaD* mutant embryos. Fewer regions where slow muscle fibers are missing are observed and slow muscle fibers are more organized than at earlier stages (compare Figs. 4D, D′ with B, B′). Many slow muscle fibers are approximately of one wild-type segment length and the dorsal–ventral stacks of fibers are more uniform than earlier in development (compare Figs. 4D, D′ with B, B′). In addition, we observe significant recovery of the three-dimensional architecture of slow muscle in *aei/deltaD* mutant embryos as seen by the clear divet (Fig. 4D″, black *) where muscle pioneers are juxtaposed to the notochord, which was absent in 20 hpf *aei/deltaD* mutant embryos (see Fig. 4B″). Thus, by 26 hpf, one can distinguish the muscle pioneers from the superficial slow muscle fibers (blue *) due to their relatively normal three-dimensional architecture and medial–lateral positioning (Fig. 4D″). We observe a similar recovery of slow muscle cell morphology and number in another Notch pathway mutant, *des/notch1a* (Supplemental Fig. 1).

Fig. 2. Three stages of segment boundary formation: (1) initial epithelial somite boundary formation, (2) transition, and (3) myotome boundary formation. Confocal micrographs (anterior left, side view); β-catenin outlines cells in red, and markers of extracellular matrix (Fibronectin, A–B) or focal adhesions (phosphorylated Focal Adhesion Kinase (Fak), C–J) are shown in green. (A–B) Initial epithelial somite boundary formation. Posterior-most somites or the approximate location of posterior-most somites in 17 hpf embryos are shown. In wild-type embryos, Fibronectin (arrow in A) localizes to initial epithelial somite boundaries flanked by epithelial border cells (arrowhead in A′ designates a border cell). When irregular initial somite boundaries form in the posterior of *aei/deltaD* mutant embryos, Fibronectin localizes to these boundaries (arrow in B) and border cells flank the boundaries (arrowhead in B′). (C–F) Transition stage. Somite 11 of 18 hpf embryos is to the left. Panels C and E are medial views, D and F are lateral views. During this stage, cells begin elongating between anterior and posterior boundaries, with medial cells elongating prior to more lateral cells (arrowhead in C′ designates a long muscle cell). Phosphorylated Fak remains concentrated at the somite boundary as it undergoes morphogenesis and begins to adopt a chevron-shaped morphology (arrow in C). More laterally (D–D′), presumptive muscle cells are still rounded but the somite boundary is beginning to adopt a chevron-shaped morphology (arrow in D). In *aei/deltaD* mutant embryos, medial muscle cells elongate (arrowhead in E′), and small regions of phosphorylated Fak concentration are observed both medially (arrow in E) and laterally (arrow in F). (G–J) Stage 3, myotome boundary formation. Somite 10 in 24 hpf embryos is to the left. Muscle cells in wild-type embryos have elongated (arrowhead in the lateral panel H′ denotes a long muscle cell) and phosphorylated Fak is heavily concentrated at myotome boundaries (arrow in G, H). Muscle cells in *aei/deltaD* mutant embryos have also elongated (arrowhead in J′ denotes a long muscle cell) and myotome boundaries demarcated by phosphorylated Fak persist throughout the medial to lateral extent of the paraxial mesoderm (arrows in I, J).

Hedgehog signaling is required for segmental recovery in aei/deltaD and des/notch1a mutant embryos

We have demonstrated that slow muscle fibers do migrate to the lateral surface in *aei/deltaD* mutant embryos and myotome boundaries persist throughout the medial to lateral extent of the paraxial mesoderm by 26 hpf. As slow muscle fibers only occupy a subset of the medial–lateral extent of the paraxial mesoderm, these data indicate that fast muscle fibers must also contribute to the formation of myotome boundaries. Because we have shown that slow muscle cells induce fast muscle cell elongation as they migrate laterally through the developing myotome (Henry and Amacher, 2004), we asked if migrating slow muscle cells in *aei/deltaD* mutant embryos might dictate the length of elongating fast muscle cells. If slow muscle cells in *aei/deltaD* mutant embryos pattern fast muscle cell elongation, we predicted that fast muscle fiber length would resemble slow muscle fiber length at the same relative dorsal–



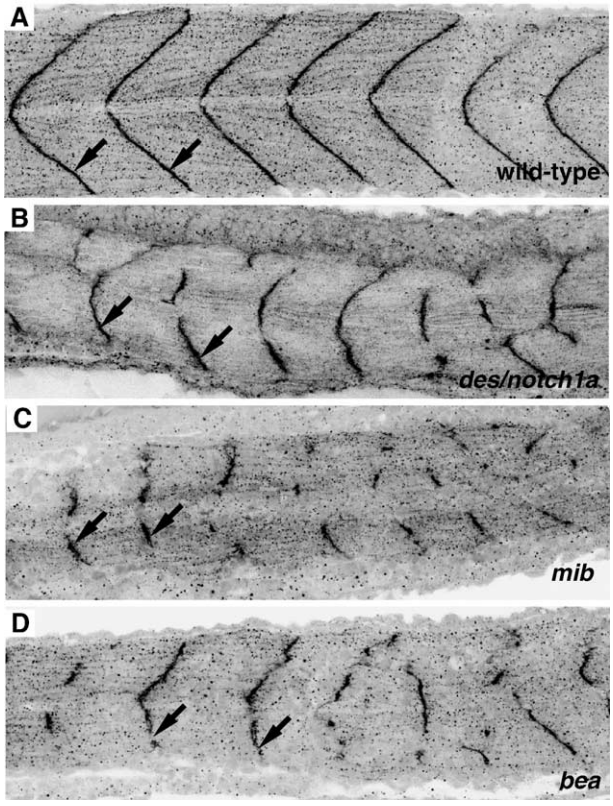


Fig. 3. Boundary recovery in known and presumptive Notch pathway mutants at 24 hpf. Phosphorylated Fak (in black; the contrast has been inverted) demarcates myotome boundaries, anterior left. Somite 10 is left in all panels except for panel D where somite 5 is to the left. (A) In wild-type embryos, chevron-shaped somite boundaries are clearly visualized by a robust accumulation of phosphorylated-Fak at the somite boundary (arrows). In *des/notch1a* (B), *beamter* (*bea*) (C), and *mind bomb* (*mib*) (D) mutant embryos, boundaries delineated by phosphorylated-Fak are seen (arrows). Although the spacing and shape of these boundaries are not normal, segmentation has significantly improved since 17 hpf (compare to Fig. 1).

ventral position. Indeed, we observe that the length of medial fast muscle cells frequently correlates with the length of slow muscle cells: i.e. fast muscle fibers are short when slow muscle fibers lateral to them are short, and fast muscle fibers are long when slow muscle fibers lateral to them are long (medial fast fiber length resembles that of lateral slow fibers 91% of the time, n = 168 fast fibers) (Fig. 5).

We hypothesized that slow muscle may be necessary for myotome boundary formation in *aei/deltaD* mutant embryos as has been suggested for another somite mutant, *fused somites/tbx24* (van Eeden et al., 1998). We tested this hypothesis by assaying myotome boundary formation in *aei/deltaD* mutant embryos incubated in cyclopamine, which blocks Hedgehog signaling and thus slow muscle specification (Barresi et al., 2000; Chen et al., 2001; Du et al., 1997; Varga et al., 2001; Wolff et al., 2003). Cyclopamine blocks the action of Smoothened, a membrane receptor essential for Hedgehog signaling (Chen et al., 2002; Ingham and McMahon, 2001). Hedgehog signaling is not necessary for the formation of initial epithelial somite boundaries (data not shown). In addition, Laminin is concentrated at myotome boundaries in wild-type and Hedgehog signaling-deficient embryos (Fig. 6, Supplemental Fig. 2, Crawford et al., 2003). Using Laminin expression to mark boundaries, we observe that myotome boundaries form by Stage 3 in *aei/deltaD* mutant embryos (Figs. 6B, B′), even posteriorly where initial epithelial somite boundaries did not form (Figs. 2I, J). Myotome boundaries also form in the anterior of cyclopamine-treated *des/notch1a* mutant embryos where initial epithelial somite boundaries form (Supplemental Fig. 2). However, when *aei/deltaD* mutant embryos are incubated in cyclopamine, myotome boundaries, as assayed by both cellular morphology and Laminin accumulation, do not form in the posterior of the embryo (Figs. 6C, C′) even though muscle cell elongation, which is initially blocked by cyclopamine treatment, has recovered by this time. Therefore, Hedgehog signaling is required for myotome boundary formation by Stage 3 in the posterior of *aei/deltaD* mutant embryos where initial epithelial somite boundaries did not form. To test whether myotome boundary recovery in other Notch signaling-deficient embryos requires Hedgehog signaling, we treated *des/notch1a* mutant embryos with cyclopamine and assayed myotome boundary recovery. As in *aei/deltaD* mutant embryos, Hedgehog signaling is required for myotome boundary formation in *des/notch1a* mutant embryos (Figs. 6D–F). Thus, our data show that Hedgehog signaling is not required for myotome boundary formation when initial epithelial somite boundaries are present, but that it is required for myotome boundary formation in Notch pathway mutant embryos in posterior regions where initial epithelial somite boundaries do not form.

We have shown that small initial epithelial somite boundaries occur in the paraxial mesoderm of *aei/deltaD* mutant embryos during initial epithelial somite boundary formation (Stage 1). Although we predict that the loss of segmentation at Stage 3 in *aei/deltaD* mutant embryos upon cyclopamine treatment is due to lack of Hedgehog-dependent slow muscle fibers, it is also possible that the weak initial boundaries that form in *aei/deltaD* mutant embryos are responsible for segmental recovery and that this process is Hedgehog-dependent. We tested this hypothesis by incubating *aei/deltaD* mutant embryos in cyclopamine and assaying for the presence of small initial epithelial somite boundaries. We observe that these small boundaries form in cyclopamine-treated mutant embryos (Fig. 7). This indicates that Hedgehog signaling is not necessary for initial weak somite boundary formation in *aei/deltaD* mutant embryos, and that the formation of initial weak somite boundaries is not sufficient for segmental recovery in the absence of Hedgehog signaling.

Somite boundaries limit myofiber elongation

Our results show that, in the absence of somite boundaries, muscle fibers can initiate the morphogenetic program of muscle cell elongation. However, given the fact that muscle cells usually elongate in between somite boundaries, we hypothesized that one function of epithelial somite boundaries may be to limit the extent to which myofibers can elongate. We utilized mosaic analysis with wild-type and *b567* mutant embryos to test this hypothesis. *b567* is a large deficiency that deletes *her1*

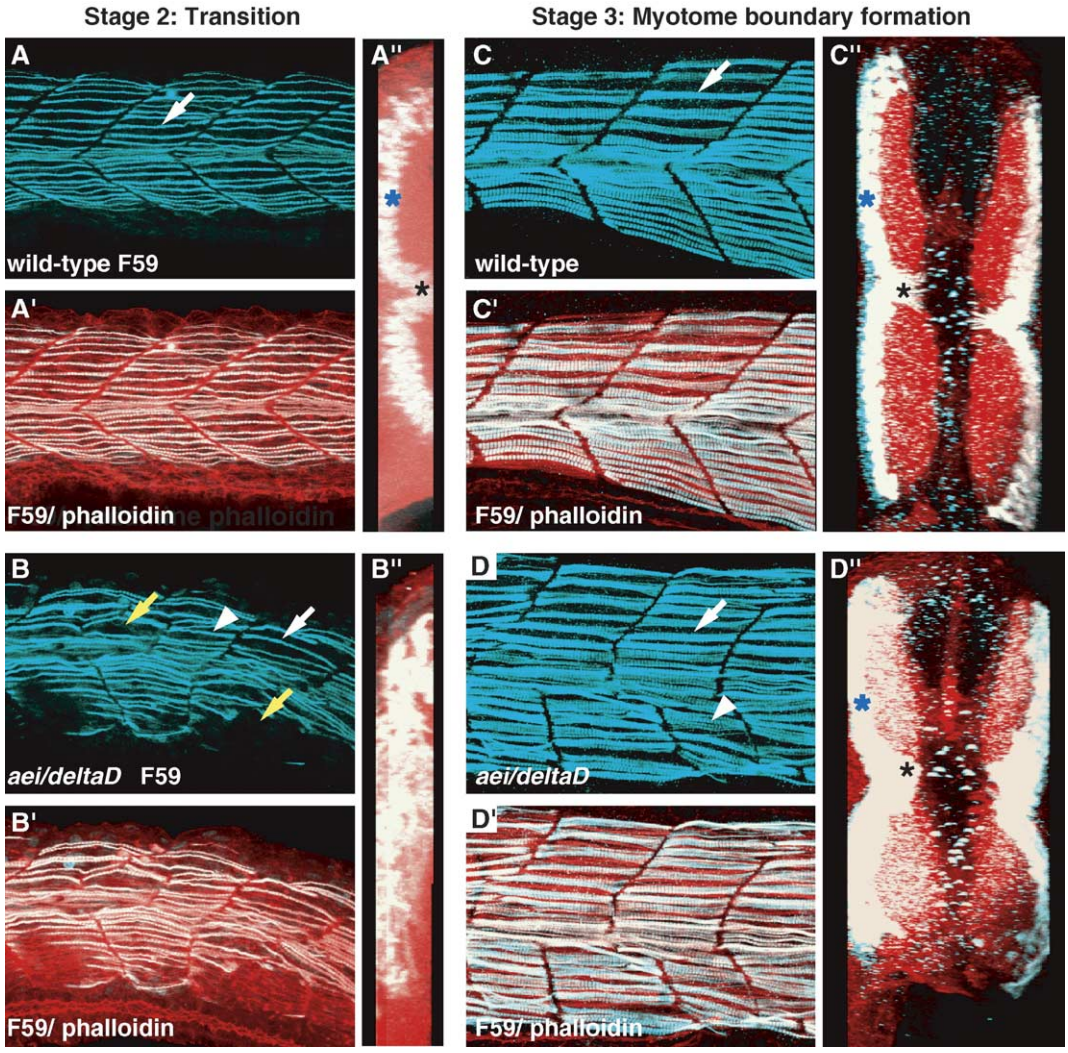


Fig. 4. Slow muscle morphogenesis is initially disrupted in the posterior of *aei/deltaD* mutant embryos but then recovers. Confocal micrographs are side views with anterior to the left, except A″–D″, which are 3 dimensionally reconstructed transverse views. F59 expression denotes slow muscle cells in blue (A–D). In prime panels (A′, B′, C′, D′), F59-expressing cells appear white due to expression of F-actin, found in all muscle cells and detected by red phalloidin staining. In wild-type embryos during the transition stage (20 hpf), a projection of slow muscle fibers shows that they are of uniform length (arrow in A; also A′, somite 10 on the left). When the same three-dimensional projection in panel A′ is rotated 90° to generate a transverse view, the three-dimensional architecture of slow muscle is apparent. Muscle pioneers are the medial-most cells (black *, medial to the right), and the migrating superficial slow fibers are more lateral (blue *) (at this stage, slow muscle fibers have migrated through most of the fast muscle mass and are nearly to the lateral surface). In stark contrast, slow muscle fibers in *aei/deltaD* mutant embryos are of varying length (B, approximate location of somite 10 is on the left) and aberrant gaps are frequently observed (yellow arrows in B; also B′). In addition, a 90° rotation of B′ to generate a transverse view shows that the normal slow muscle three-dimensional architecture is severely disrupted. Slow muscle fibers are scattered throughout the paraxial mesoderm and there is no clear distinction between the location of muscle pioneers and the superficial slow fibers. However, this defect in slow muscle morphogenesis in *aei/deltaD* mutant embryos significantly recovers by 26 hpf (compare C″ and D″). In wild-type embryos at the myotome boundary stage (26 hpf), slow muscle cells are of uniform length (C, C′, somite 5 is on the left), and a transverse view of a different embryo shows clear three-dimensional architecture of slow muscle with muscle pioneers medial (black *) and superficial slow muscle fibers lateral (blue *). In *aei/deltaD* mutant embryos at this stage, a projection of slow muscle fibers indicates that mostly organized dorsal ventral stacks of slow muscle cells form (D, somite 10 is on the left). The length of slow muscle fibers is more regular, although both longer (arrow) and shorter (arrowhead) fibers are observed (see also D′, a projection of D with phalloidin in red to visualize all cells). Significantly, gaps in slow muscle fibers are rarely observed. In addition, three-dimensional reconstruction of a different *aei/deltaD* mutant embryo that has been rotated 90° to generate a transverse view shows significant recovery of the three-dimensional architecture of slow muscle (compare to B″). A divet in the slow muscle can be seen at the location of the muscle pioneers (black *), and most slow muscle has migrated to occupy the most superficial layer of the paraxial mesoderm (blue *).

and *her7* (two transcriptional repressors induced by Notch signaling) and severely disrupts somite boundary formation (Henry et al., 2002). Although strong somite boundaries form with a 1 1/2 to 2 segment periodicity in *her1*- and *her7*-deficient embryos (generated using antisense morpholino oligomer injections), boundary formation in *b567* mutant embryos is more severely disrupted (Henry et al., 2002). Muscle cells in *b567* mutant embryos are frequently longer than wild-type muscle cells (Figs. 8A, B). When *b567* mutant cells are transplanted into wild-type hosts, we find that *b567* mutant muscle cells are of normal length, independent of the number of cells transplanted (Figs. 8G, H) (n = 15 embryos, 139 cells, 2 experiments). In the reciprocal transplant, wild-type cells placed into *b567* mutant hosts resemble *b567* mutant

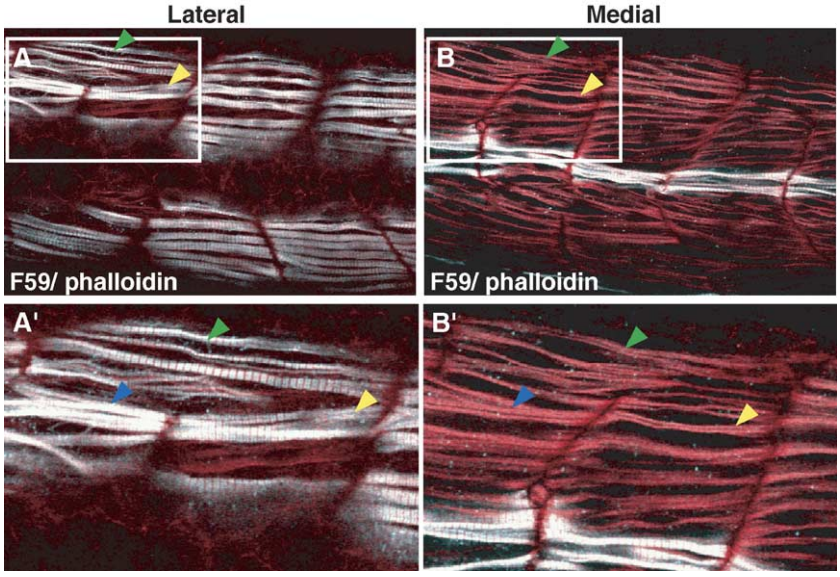


Fig. 5. Fast muscle fiber organization mimics that of slow muscle in the *aei/deltaD* myotome. Confocal micrographs (anterior left, dorsal top) during Stage 3 (26 hpf) of an *aei/deltaD* mutant embryo stained with F59 (white) and phalloidin (red); the approximate location of somite 10 is on the left. At this stage, slow muscle fibers have migrated to the lateral extent of the myotome, and fast muscle fibers are medial to slow fibers. Panels A and B are single confocal micrographs from a medial to lateral Z-series that demonstrates that medial fast fiber length mimics lateral slow muscle fiber length. Panel A is a lateral section containing superficial slow fibers (white), and panel B is a medial section containing muscle pioneers (white). Prime (′) panels are higher magnification views of the boxed regions in A and B. (A) Many lateral superficial slow muscle fibers are organized and have elongated to occupy one wild-type segment length (yellow arrowhead), but some longer fibers (green arrowhead) are observed. (A′) At a higher magnification, some abnormally long slow fibers (green arrowhead) are observed, but other fibers are more normal in length (blue and yellow arrowheads). (B, B′) Medially, the red fast fibers (those lacking the F59 slow muscle myosin antigen), have the same organization as the slow fibers that lie adjacent (lateral) to them in the medio-lateral plane (compare to A, A′). Specifically, in the higher magnification view (B′), abnormally long fast fibers (green arrowhead) are observed where abnormally long slow fibers are observed and shorter fast fibers are observed where short slow fibers were observed (blue and yellow arrowheads). Thus, the length of the medial fast fibers correlates with the lengths of the superficial slow fibers.

cells (Figs. 8I, J) (n = 18 embryos, 186 cells, 2 experiments). Thus, transplanted wild-type or *b567* mutant fast muscle cells are of normal length if they elongate in between two closely spaced boundaries, but are abnormally long otherwise.

Discussion

Segmentation of the body plan is an integral aspect of development. In vertebrates, perhaps the most obvious segmental structures seen in early development are the somites that form from the presomitic mesoderm. Notch signaling is necessary for normal somite formation in mouse, chicken, zebrafish, and *Xenopus* (Bessho et al., 2001; Dale et al., 2003; Evrard et al., 1998; Henry et al., 2002; Holley et al., 2000, 2002; Itoh et al., 2003; Jen et al., 1997; Koizumi et al., 2001; Oates and Ho, 2002; Zhang and Gridley, 1998). However, some zebrafish Notch pathway mutants are homozygous viable when reared in the lab, implying that mesodermal segmentation recovers in these embryos. In order to understand segmental recovery in Notch pathway mutant embryos, we have addressed the interplay between somite boundaries and slow muscle fibers.

Three stages of segment boundary development

We currently define three stages of development of segment boundaries in the zebrafish embryo (Fig. 2). The first stage is the formation of initial epithelial somite boundaries from the presomitic mesoderm. These boundaries are formed by the alignment and epithelialization of presumptive border cells (Henry et al., 2000) and focal adhesion components localize to these initial epithelial somite boundaries (Crawford et al., 2003; Henry et al., 2001). The second stage is a transition stage. During this stage, migrating slow muscle cells induce fast muscle cell elongation (Henry and Amacher, 2004). The somite boundary is also undergoing morphogenesis as it becomes more chevron-shaped. One inherent aspect of the transition stage is that the initial epithelial somite boundary is maintained through time. The third stage, myotome boundary formation, is marked by robust localization of focal adhesion and dystroglycan components to a chevron-shaped boundary that flanks fully elongated muscle fibers (Bassett et al., 2003; Crawford et al., 2003; Guyon et al., 2003; Parsons et al., 2002). These observations have led us to propose a model (Fig. 9), which will be discussed subsequently in more detail.

Slow muscle morphogenesis in aei/deltaD mutant embryos

Due to the fact that slow muscle is specified normally in *aei/deltaD* mutant embryos (van Eeden et al., 1996), it has been thought that slow muscle development in these embryos is also normal. Although slow muscle is specified in *aei/deltaD* mutant embryos, we show that muscle fiber length is variable and gaps in slow muscle are observed, particularly during Stage 2 (Fig. 4). In addition, we show that the normal slow muscle three-dimensional architecture is disrupted at this stage.

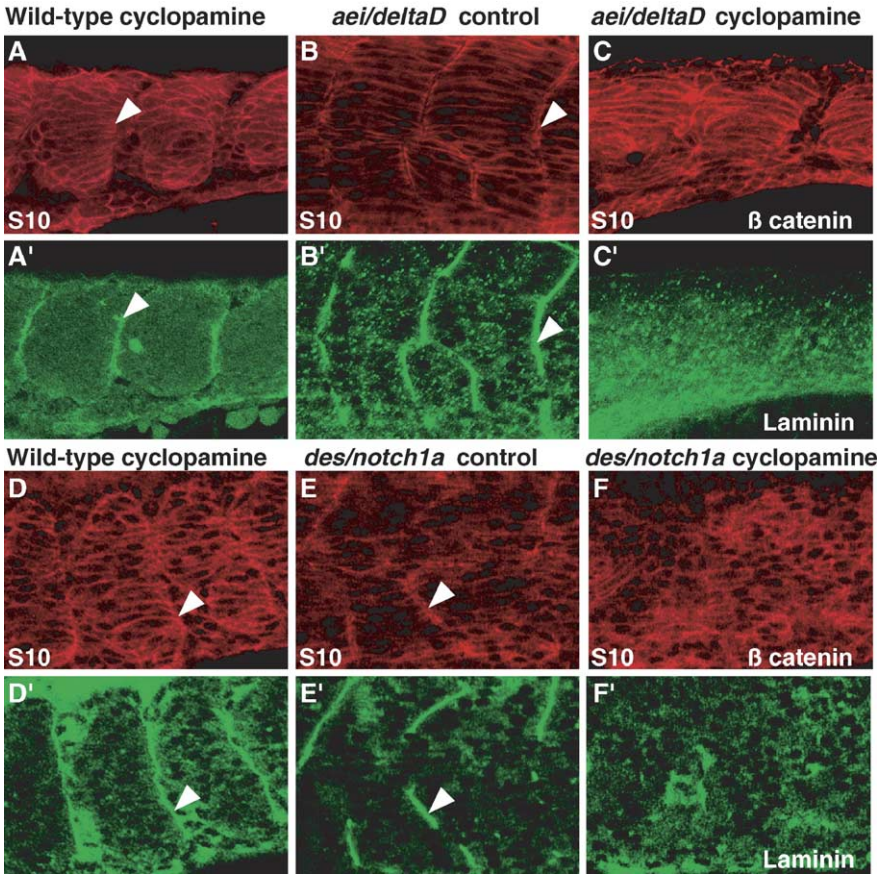


Fig. 6. Irregular myotome boundary formation in *aei/deltaD* mutant embryos requires Hedgehog signaling. All panels are confocal micrographs (side views, anterior left) of 24 hpf embryos; β-catenin expression is in red, Laminin expression is in green. Micrographs of control and cyclopamine-treated embryos are at comparable anterior–posterior and medial–lateral positions with somite 10 or the approximate location of somite 10 to the left in all panels. In cyclopamine-treated wild-type siblings from *aei/deltaD* and *des/notch1a* intercrosses, β-catenin reveals myotome boundaries (A, D) and Laminin localizes at myotome boundaries (A′, D′, arrowheads). In control *aei/deltaD* (B, B′) and *des/notch1a* (E, E′) mutant embryos, irregular boundaries are visualized by robust expression of β-catenin (B, E, arrowheads) and Laminin (B′, E′ arrowheads). However, in cyclopamine-treated *aei/deltaD* mutant embryos (C, C′; n = 24 mutant embryos, 4 experiments) and cyclopamine-treated *des/notch1a* mutant embryos (F, F′; n = 11 mutant embryos, 3 experiments), boundaries do not form (C, F) and expression of Laminin is diffuse (C′, F′).

However, slow muscle morphology significantly recovers by myotome boundary formation, Stage 3. At this point in time, *aei/deltaD* mutant slow muscle fibers are more consistent in length and gaps in slow muscle fibers are not observed. Furthermore, slow muscle three-dimensional architecture is significantly recovered. It will be interesting to determine the cellular and molecular mechanisms that underlie this dramatic recovery.

Hedgehog signaling is necessary for somite boundary morphogenesis in aei/deltaD and des/notch1a mutant embryos

We have shown that Hedgehog signaling is necessary for recovery of segmentation in *aei/deltaD* and *des/notch1a* mutant embryos (Fig. 6). Another zebrafish mutant, *fused somites/tbx24* (*fss/tbx24*) also exhibits defects in initial somite boundary formation and is homozygous viable (Nikaido et al., 2002; van Eeden et al., 1996). At 72 h, irregular boundaries are observed in the paraxial mesoderm of *fss/tbx24* mutant embryos (van Eeden et al., 1998). Utilizing double mutant analysis, it was shown that the formation of these irregular boundaries is dependent upon normal Hedgehog signaling, suggesting that slow muscle might pattern segmental recovery in *fss/tbx24* mutant embryos (van Eeden et al., 1998).

We have shown that migrating slow muscle induces fast muscle cell elongation (Henry and Amacher, 2004). In this study, we show that the pattern of fast muscle fiber elongation generally mimics the pattern of slow muscle fibers that elongate to aberrant lengths in the absence of somite boundaries (Fig. 5). Blocking slow muscle induction with cyclopamine treatment also blocks organized fast muscle fiber elongation and the formation of myotome boundaries in *aei/deltaD* and *des/notch1a* mutant embryos (Fig. 6). Taken together, these data strongly suggest that slow muscle significantly contributes to patterning segmental recovery in Notch pathway mutant embryos. However, it is possible that the Engrailed-expressing medial fast fibers are responsible for or contribute to segmental recovery, as cyclopamine treatment also blocks the specification of this cell type (Wolff et al., 2003). It is interesting to note that, as of yet, no single mutation that disrupts myotome boundary formation along the entire anterior–posterior axis has been isolated (Holley et al., 2000,

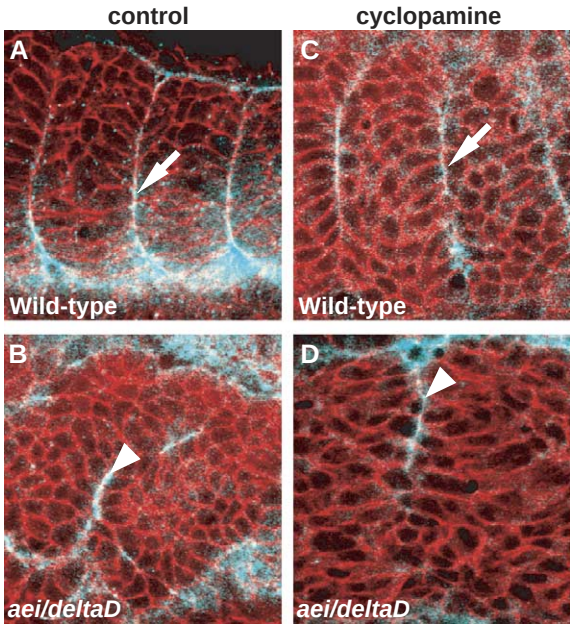


Fig. 7. Hedgehog signaling is not required for the formation of initial weak epithelial somite boundaries in *aei/deltaD* mutant embryos. All panels (side views, anterior left) show the most recently formed somite boundaries in 17 hpf embryos; β-catenin is shown in red and Laminin is shown in blue. In control (A) and cyclopamine-treated (B) wild-type embryos, Laminin concentrates at somite boundaries (arrows). (B) In control *aei/deltaD* mutant embryos (C), weak attempts at boundary formation are visible as clefts that stain with Laminin (arrowhead) and similar weak boundaries are observed in cyclopamine-treated *aei/deltaD* mutant embryos (D, arrowhead).

2002; Itoh et al., 2003). Here, we show that when both Hedgehog and Notch pathways are disrupted, myotome boundaries do not form. Similar to this result, myotome boundary formation is largely abrogated in *aei/deltaD; integrinα5* double mutants (Julich et al., 2005), in *fused somites* (*fss*)*; sonic you* (*syu*)*/sonic hedgehog* double mutants (van Eeden et al., 1998), and in embryos deficient for both *deltaC* and *her7* (Oates et al., 2005). Taken together, these results raise the possibility that there are multiple mechanisms contributing to myotome boundary formation, thus generating the observed robustness of the process. It is interesting to speculate that Hedgehog signaling may be critical for Integrin-mediated processes; *integrinα5* is provided maternally, but also is zygotically expressed in slow muscle precursor cells and functions in anterior myotome boundary formation (Julich et al., 2005; Koshida et al., 2005) and redundantly with the Notch pathway for posterior myotome boundary formation (Julich et al., 2005). Our data suggest that slow muscle migration is an alternate mechanism to achieve myotome boundary formation when initial epithelial somite boundaries do not form.

Somite boundaries limit myofiber elongation

Our results indicate that both fast and slow muscle cells begin the morphogenetic program of cell elongation in the absence of initial epithelial somite boundaries. However, fast- and slow-twitch muscle cells may utilize different mechanisms to limit their length. Posterior slow muscle cells in 24 hpf *aei/deltaD* mutant embryos are frequently about one segment length even though initial epithelial somite boundaries did not form. This observation suggests that slow muscle cells can regulate their length even in the absence of initial epithelial somite boundaries and may impose this length upon fast muscle cells during their lateralward migration. To test the hypothesis that initial epithelial somite boundaries do modulate muscle cell length, we made genetic mosaics using *b567* mutant embryos, which lack *her1* and *her 7* (transcriptional repressors transcribed in response to Notch signaling). In *b567* mutant embryos, muscle cells are frequently longer than those in wild-type embryos (Henry et al., 2002; Fig. 8). We show that one factor limiting muscle cell elongation in *b567* mutant embryos is the presence of an initial epithelial somite boundary and that muscle fibers elongate to varying lengths in the absence of initial epithelial somite boundaries (Fig. 8). This observation indicates that the formation of reiterated somite boundaries may pattern uniform muscle cell length. Furthermore, this result suggests the hypothesis that muscle cells keep elongating until they reach the extracellular matrix at the somite boundary and then adhere to the matrix and stop elongating.

Mechanisms of myofiber elongation and boundary capture

The mechanism of muscle fiber elongation is not well understood. Fast muscle cell fusion in zebrafish is observed by 24 hpf, but it is not known if fusion contributes to myofiber elongation (Kimmel and Warga, 1987; Roy et al., 2001). However, parallels can be drawn with elegant models of notochord/neural plate cell intercalation (Keller et al., 2000). We hypothesize that somitic cells gain bipolar polarity as they extend in the anterior to posterior dimension. Cells may extend by crawling on cells beneath them (cell–cell traction model of cell intercalation), and/or by crawling on small amounts of extracellular matrix that may exist between the cells (cell–matrix model of cell intercalation) (Keller et al., 2000). In fact, the cell–matrix adhesion molecules Paxillin and Fak, and the cell–cell adhesion molecules M- and N-cadherin, are expressed in zebrafish somitic cells (Cortes et al., 2003; Crawford et al., 2003). However, M- and N-cadherin, although required for normal slow muscle migration, do not appear to be required for muscle cell elongation (Cortes et al., 2003). Clearly, a more detailed analysis of cell–cell adhesions, cell–matrix adhesions, and cell fusion is needed in order to fully understand the mechanism of myofiber elongation.

How do somite boundaries limit the length of myofibers? In the context of intercalating notochord cells, it has been postulated that the notochord–somite boundary functions to "capture" intercalating notochord cells and prevent them from extending into the paraxial mesoderm (Keller et al., 2000). We hypothesize that a similar boundary capture mechanism may function in myofiber elongation. Fibronectin and Laminin localize to zebrafish somite boundaries (Crawford et al., 2003), indicating extracellular matrix deposition at zebrafish somite boundaries. When an elongating myofiber contacts a somite boundary, it may form strong adhesions to the matrix,

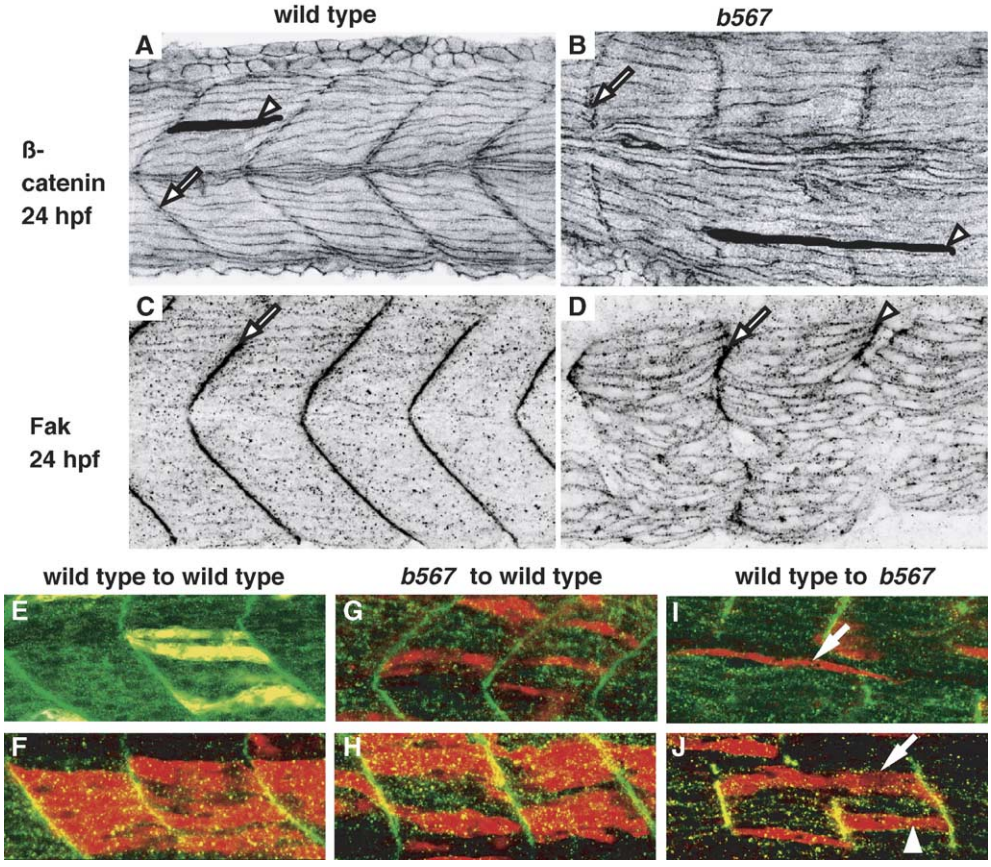


Fig. 8. Somite boundaries limit myofiber length. All panels are confocal micrographs (side views, anterior left) of 24 hpf embryos. Wild-type and *b567* (a deficiency that deletes *her1* and *her7*, see text for discussion) mutant embryos are shown at comparable anterior–posterior and medial–lateral positions. (A) β-catenin expression (contrast has been inverted so that cells are outlined in black) in wild-type embryos shows chevron-shaped myotome boundaries (arrow). (B) β-catenin expression in *b567* mutant embryos shows that poor boundaries do form (arrow). Cells traced and filled black in Adobe Photoshop show that myofibers in wild-type embryos are normal length (A, arrowhead), but myofibers in *b567* mutant embryos are frequently longer (B, arrowhead). (C) phospho-Fak expression (contrast inverted) in wild-type embryos delineates chevron-shaped somites (arrow). (D) phospho-Fak expression in *b567* embryos shows poor myotome boundary formation (arrowhead, arrow points to a fairly robust somite boundary). (E–J) Mosaic analysis with transplanted rhodamine–dextran-labeled cells in red and phospho-Fak expression in green. Transplanted cells in panel E are yellow due to the wavelengths of the secondary antibodies used in that experiment. (E, F) Wild-type cells transplanted into wild-type hosts are of normal length. (G, H) Both small (G) and large (H) groups of *b567* mutant cells transplanted to wild-type hosts are of normal length. (I) An isolated wild-type cell transplanted into a *b567* mutant host (arrow) is aberrantly long. (J) Wild-type cells located between normally spaced boundaries in *b567* mutant hosts are of normal length (arrowhead) whereas a cell between distantly spaced boundaries is unusually long (arrow).

prohibiting further movement. In support of this hypothesis, our genetic mosaic analysis shows that, in the absence of normal somite boundary formation, muscle cells elongate until they reach a somite boundary.

Interactions between somite boundaries, slow muscle cells, and fast muscle cells

We have investigated the relationships between epithelial somite boundary formation, muscle fiber formation, and myotome formation. This study, along with other recent work, allows us to propose the following model (Fig. 9). In a wild-type embryo, segmentation and muscle development proceed in a stereotypical manner. Hedgehog signaling specifies slow muscle progenitors even prior to overt somite boundary formation (Barresi et al., 2000; Du et al., 1997; Wolff et al., 2003). Next, Notch signaling, along with other signaling pathways, plays a significant role in somite boundary placement (Holley and Takeda, 2002; Pourquie, 2003a). During the transition stage, muscle cells begin elongating and somite boundaries limit the elongation of muscle fibers. Superficial slow muscle fibers then migrate laterally through the fast muscle and instruct fast muscle cells to elongate in their wake (Henry and Amacher, 2004). Finally, the somite boundary matures into a myotome, becoming chevron-shaped and extremely rich in extracellular matrix as visualized by the robust concentration of Laminin, Fibronectin, Fak, and Paxillin, components of the dystroglycan complex, and periostin (Bassett et al., 2003; Crawford et al., 2003; Henry et al., 2001; Kudo et al., 2004; Parsons et al., 2002).

When Notch signaling is disrupted, embryos lack regularly spaced epithelial somite boundaries, and slow muscle fibers elongate to varying lengths and migrate abnormally towards the lateral surface. However, by 24–26 hpf, slow muscle morphogenesis has significantly recovered and robust, albeit irregular, myotome boundaries have formed. Because Hedgehog signaling is required for recovery in both *aei/deltaD* and *des/notch1a* mutants, we hypothesize that slow muscle fibers

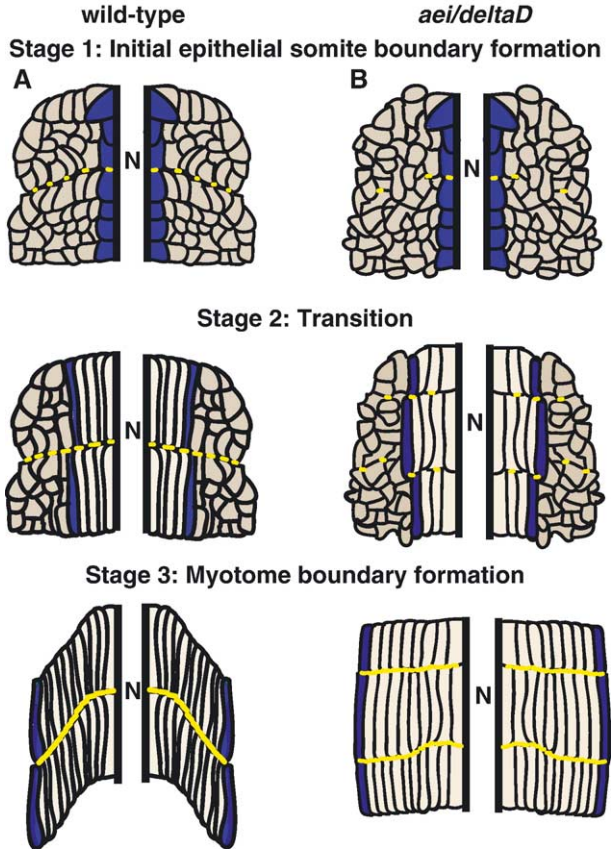


Fig. 9. Model of the three stages of segment boundary formation and interactions between somite boundaries and muscle cells. All panels are dorsal views, anterior towards the top, N denotes notochord. Slow muscle cells are indicated in blue, rounded fast muscle precursor cells in brown, elongated fast muscle cells in light tan, and extracellular matrix/focal adhesion proteins in yellow. In wild-type embryos (A), initial epithelial somite boundary formation is accompanied by the concentration of extracellular matrix/focal adhesion proteins at the somite boundary. During the transition stage, slow muscle cells elongate in the anterior–posterior dimension until they hit the epithelial somite boundaries, and at the same time, begin their lateral migration. Thus, in this sense, somite boundaries pattern muscle segmentation. The medial to lateral migration of slow muscle cells induces fast muscle cell elongation (Henry and Amacher, 2004). By the time the myotome has formed, the myotome boundary is exceedingly rich in extracellular matrix/focal adhesion/dystroglycan complex components. Notch signaling is required for normal initial epithelial somite boundary formation (B), thus, in *aei/deltaD* mutant embryos at this stage, extracellular matrix deposition reflects aberrant epithelial somite boundary formation. During the transition stage, *aei/deltaD* mutant slow muscle cells elongate. However, since epithelial somite boundaries that normally coordinate elongation are lacking, slow muscle cell length is variable. These slow muscle fibers do, however, migrate laterally and induce fast muscle cell elongation. We hypothesize that migrating slow muscle fibers pattern the extent to which fast muscle can elongate. Therefore, by the time the myotome has formed, fast muscle cells have elongated in coordinated medio-lateral stacks. The coordinated elongation of *aei/deltaD* superficial slow muscle and the underlying fast fibers create boundaries in the paraxial mesoderm that develop into bona fide mature myotome boundaries that are rich in extracellular matrix/focal adhesion/dystroglycan complex components, despite the absence of early epithelial somite boundaries.

in Notch pathway mutant embryos play an active, instructive role. One of the extraordinary aspects of the medial to lateral migration of slow muscle is that the slow muscle cells migrate as elongated fibers (Cortes et al., 2003; Devoto et al., 1996). Because the extent of fast muscle fiber elongation correlates with that of slow muscle fibers, we hypothesize that the migrating superficial slow muscle fibers in *aei/deltaD* mutant embryos not only instruct fast fibers to elongate but also pattern the fast fiber length to roughly equal the length of the instructing slow muscle fiber. As the migrating mutant slow muscle fibers are not of uniform length, they instruct fast fibers to elongate to varying lengths during their migration, resulting in the formation of fissures in the paraxial mesoderm between fibers. Because these fissures are flanked by bona fide muscle fibers, they develop into mature myotome boundaries that contain robust concentrations of Fak and Laminin.

In cyclopamine-treated *aei/deltaD* and *des/notch1a* mutant embryos, Hedgehog signaling is blocked, slow muscle fibers are not specified, and somite boundaries do not form by 24–26 hpf. The weak initial epithelial somite boundaries observed in control *aei/deltaD* mutant embryos are also observed in cyclopamine-treated *aei/deltaD* mutant embryos, indicating that weak boundaries are not sufficient to pattern segmental recovery. Taken together, our data suggest that slow muscle fiber elongation and migration do pattern relatively organized fast muscle fiber elongation even in the absence of normal epithelial somite boundary formation.

We have shown that the medial to lateral migration of slow muscle fibers instigates timely fast muscle fiber elongation. However, later in development (24–26 hpf), fast muscle fibers do eventually elongate even in the absence of Hedgehog signaling (Henry and Amacher, 2004). In this paper, we show that fast fibers have also elongated in cyclopamine-treated *aei/deltaD* and *des/notch1a* mutant embryos by 24–26 hpf, but that elongation is clearly not sufficient to pattern myotome boundary formation. This insufficiency may be due to the fact that the fast fibers are not elongating in a coordinated fashion since instructive slow muscle fibers are lacking. However, it is also possible that slow muscle fibers may directly influence processes required for somite boundary recovery such as the secretion of a rich extracellular matrix or that Hedgehog signaling is directly required for myotome boundary formation in Notch pathway mutant embryos.

Acknowledgments

We thank Jen St. Hilaire and Emily Janus for excellent zebrafish care and Jen Anderson for technical support. We thank all members of the laboratory, especially Kariena Dill, Tina Han, and Dave Daggett for helpful discussion and critical reading of the manuscript. We appreciate John Wallingford and Jeremy Reiter for their critical evaluation of earlier versions. We thank Steve Devoto for helpful advice and Andy Oates for *after eight/deltaD* mutant fish. S.L.A. thanks many colleagues at the University of Oregon who participated in a group screening effort resulting in the isolation of des^{b638} and bea^{b663}. We thank Holly Aaron and the Molecular Imaging Center at Berkeley for technical advice and Frank Stockdale for the F59 antibody. This work was supported by an NIH grant (GM61952-01) and a Pew Scholar award to S.L.A. C.A.H. was a Miller Fellow in the

Department of Molecular and Cell Biology at the University of California, Berkeley.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2005.08.049.

References

Amacher, S.L., Kimmel, C.B., 1998. Promoting notochord fate and repressing muscle development in zebrafish axial mesoderm. Development 125, 1397–1406.

Barresi, M.J., Stickney, H.L., Devoto, S.H., 2000. The zebrafish *slow-muscle-omitted* gene product is required for Hedgehog signal transduction and the development of slow muscle identity. Development 127, 2189–2199.

Barresi, M.J., D'Angelo, J.A., Hernandez, L.P., Devoto, S.H., 2001. Distinct mechanisms regulate slow-muscle development. Curr. Biol. 11, 1432–1438.

Bassett, D.I., Bryson-Richardson, R.J., Daggett, D.F., Gautier, P., Keenan, D.G., Currie, P.D., 2003. Dystrophin is required for the formation of stable muscle attachments in the zebrafish embryo. Development 130, 5851–5860.

Bessho, Y., Sakata, R., Komatsu, S., Shiota, K., Yamada, S., Kageyama, R., 2001. Dynamic expression and essential functions of *Hes7* in somite segmentation. Genes Dev. 15, 2642–2647.

Blagden, C.S., Currie, P.D., Ingham, P.W., Hughes, S.M., 1997. Notochord induction of zebrafish slow muscle mediated by Sonic hedgehog. Genes Dev. 11, 2163–2175.

Chen, W., Burgess, S., Hopkins, N., 2001. Analysis of the zebrafish *smoothened* mutant reveals conserved and divergent functions of hedgehog activity. Development 128, 2385–2396.

Chen, J.K., Taipale, J., Cooper, M.K., Beachy, P.A., 2002. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev. 16, 2743–2748.

Cortes, F., Daggett, D., Bryson-Richardson, R.J., Neyt, C., Maule, J., Gautier, P., Hollway, G.E., Keenan, D., Currie, P.D., 2003. Cadherin-mediated differential cell adhesion controls slow muscle cell migration in the developing zebrafish myotome. Dev. Cell 5, 865–876.

Coutelle, O., Blagden, C.S., Hampson, R., Halai, C., Rigby, P.W., Hughes, S.M., 2001. Hedgehog signalling is required for maintenance of *myf5* and *myoD* expression and timely terminal differentiation in zebrafish adaxial myogenesis. Dev. Biol. 236, 136–150.

Crawford, B.D., Henry, C.A., Clason, T.A., Becker, A.L., Hille, M.B., 2003. Activity and distribution of paxillin, focal adhesion kinase, and cadherin indicate cooperative roles during zebrafish morphogenesis. Mol. Biol. Cell 14, 3065–3081.

Currie, P.D., Ingham, P.W., 1996. Induction of a specific muscle cell type by a Hedgehog-like protein in zebrafish. Nature 382, 452–455.

Currie, P.D., Ingham, P.W., 1998. The generation and interpretation of positional information within the vertebrate myotome. Mech. Dev. 73, 3–21.

Dale, J.K., Maroto, M., Dequeant, M.L., Malapert, P., McGrew, M., Pourquie, O., 2003. Periodic Notch inhibition by Lunatic fringe underlies the chick segmentation clock. Nature 421, 275–278.

Denetclaw Jr., W.F., Christ, B., Ordahl, C.P., 1997. Location and growth of epaxial myotome precursor cells. Development 124, 1601–1610.

Denetclaw Jr., W.F., Berdougo, E., Venters, S.J., Ordahl, C.P., 2001. Morphogenetic cell movements in the middle region of the dermomyotome dorsomedial lip associated with patterning and growth of the primary epaxial myotome. Development 128, 1745–1755.

Devoto, S.H., Melancon, E., Eisen, J.S., Westerfield, M., 1996. Identification of separate slow and fast muscle precursor cells in vivo, prior to somite formation. Development 122, 3371–3380.

Du, S.J., Devoto, S.H., Westerfield, M., Moon, R.T., 1997. Positive and negative regulation of muscle cell identity by members of the *hedgehog* and TGF-β gene families. J. Cell. Biol. 139, 145–156.

Dubois, G.M., Haftek, Z., Crozet, C., Garrone, R., Le Guellec, D., 2002. Structure and spatio temporal expression of the full length DNA complementary to RNA coding for α2 type I collagen of zebrafish. Gene 294, 55–65.

Evrard, Y.A., Lun, Y., Aulehla, A., Gan, L., Johnson, R.L., 1998. *lunatic fringe* is an essential mediator of somite segmentation and patterning. Nature 394, 377–381.

Gajewski, M., Sieger, D., Alt, B., Leve, C., Hans, S., Wolff, C., Rohr, K.B., Tautz, D., 2003. Anterior and posterior waves of cyclic *her1* gene expression are differentially regulated in the presomitic mesoderm of zebrafish. Development 130, 4269–4278.

Gray, M., Moens, C.B., Amacher, S.L., Eisen, J.S., Beattie, C.E., 2001. Zebrafish *deadly seven* functions in neurogenesis. Dev. Biol. 237, 306–323.

Guyon, J.R., Mosley, A.N., Zhou, Y., O'Brien, K.F., Sheng, X., Chiang, K., Davidson, A.J., Volinski, J.M., Zon, L.I., Kunkel, L.M., 2003. The dystrophin associated protein complex in zebrafish. Hum. Mol. Genet. 12, 601–615.

Henry, C.A., Amacher, S.L., 2004. Zebrafish slow muscle cell migration induces a wave of fast muscle morphogenesis. Dev. Cell 7, 917–923.

Henry, C.A., Hall, L.A., Hille, M.B., Solnica-Krezel, L., Cooper, M.S., 2000. Somites in zebrafish doubly mutant for *knypek* and *trilobite* form without internal mesenchymal cells or compaction. Curr. Biol. 10, 1063–1066.

Henry, C.A., Crawford, B.D., Yan, Y.L., Postlethwait, J., Cooper, M.S., Hille, M.B., 2001. Roles for zebrafish focal adhesion kinase in notochord and somite morphogenesis. Dev. Biol. 240, 474–487.

Henry, C.A., Urban, M.K., Dill, K.K., Merlie, J.P., Page, M.F., Kimmel, C.B., Amacher, S.L., 2002. Two linked *hairy/Enhancer of split*-related zebrafish genes, *her1* and *her7*, function together to refine alternating somite boundaries. Development 129, 3693–3704.

Hirsinger, E., Stellabotte, F., Devoto, S.H., Westerfield, M., 2004. Hedgehog signaling is required for commitment but not initial induction of slow muscle precursors. Dev. Biol. 275, 143–157.

Holley, S.A., Takeda, H., 2002. Catching a wave: the oscillator and wavefront that create the zebrafish somite. Semin. Cell Dev. Biol. 13, 481–488.

Holley, S.A., Geisler, R., Nusslein-Volhard, C., 2000. Control of *her1* expression during zebrafish somitogenesis by a Delta-dependent oscillator and an independent wave-front activity. Genes Dev. 14, 1678–1690.

Holley, S.A., Julich, D., Rauch, G.J., Geisler, R., Nusslein-Volhard, C., 2002. *her1* and the *Notch* pathway function within the oscillator mechanism that regulates zebrafish somitogenesis. Development 129, 1175–1183.

Ingham, P.W., McMahon, A.P., 2001. Hedgehog signaling in animal development: paradigms and principles. Genes Dev. 15, 3059–3087.

Itoh, M., Kim, C.H., Palardy, G., Oda, T., Jiang, Y.J., Maust, D., Yeo, S.Y., Lorick, K., Wright, G.J., Ariza-McNaughton, L., Weissman, A.M., Lewis, J., Chandrasekharappa, S.C., Chitnis, A.B., 2003. Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev. Cell 4, 67–82.

Jen, W.C., Wettstein, D., Turner, D., Chitnis, A., Kintner, C., 1997. The Notch ligand, X-Delta-2, mediates segmentation of the paraxial mesoderm in *Xenopus* embryos. Development 124, 1169–1178.

Jiang, Y.J., Smithers, L., Lewis, J., 1998. Vertebrate segmentation: the clock is linked to Notch signalling. Curr. Biol. 8, R868–R871.

Jowett, T., 1999. Analysis of protein and gene expression. Methods Cell Biol. 59, 63–85.

Julich, D., Geisler, R., Holley, S.A., 2005. Integrinα5 and Delta/Notch signaling have complementary spatiotemporal requirements during zebrafish somitogenesis. Dev. Cell 8, 575–586.

Kahane, N., Cinnamon, Y., Kalcheim, C., 1998. The cellular mechanism by which the dermomyotome contributes to the second wave of myotome development. Development 125, 4259–4271.

Kalcheim, C., Cinnamon, Y., Kahane, N., 1999. Myotome formation: a multistage process. Cell Tissue Res. 296, 161–173.

Keller, R., Davidson, L., Edlund, A., Elul, T., Ezin, M., Shook, D., Skoglund,

P., 2000. Mechanisms of convergence and extension by cell intercalation. Philos. Trans. R Soc. Lond., B Biol. Sci. 355, 897–922.

Kielbowna, L., 1981. The formation of somites and early myotomal myogenesis in *Xenopus laevis*, *Bombina variegata* and *Pelobates fuscus*. J. Embryol. Exp. Morphol. 64, 295–304.

Kimmel, C.B., Warga, R.M., 1987. Cell lineages generating axial muscle in the zebrafish embryo. Nature 327, 234–237.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Koizumi, K., Nakajima, M., Yuasa, S., Saga, Y., Sakai, T., Kuriyama, T., Shirasawa, T., Koseki, H., 2001. The role of presenilin 1 during somite segmentation. Development 128, 1391–1402.

Koshida, S., Kishimoto, Y., Ustumi, H., Shimizu, T., Furutani-Seiki, M., Kondoh, H., Takada, S., 2005. Integrinα5-dependent Fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos. Dev. Cell 8, 587–598.

Kudo, H., Amizuka, N., Araki, K., Inohaya, K., Kudo, A., 2004. Zebrafish periostin is required for the adhesion of muscle fiber bundles to the myoseptum and for the differentiation of muscle fibers. Dev. Biol. 267, 473–487.

Kulesa, P.M., Fraser, S.E., 2002. Cell dynamics during somite boundary formation revealed by time-lapse analysis. Science 298, 991–995.

Lewis, K.E., Currie, P.D., Roy, S., Schauerte, H., Haffter, P., Ingham, P.W., 1999. Control of muscle cell-type specification in the zebrafish embryo by Hedgehog signalling. Dev. Biol. 216, 469–480.

Neff, A.W., Malacinski, G.M., Chung, H.M., 1989. Amphibian (urodele) myotomes display transitory anterior/posterior and medial/lateral differentiation patterns. Dev. Biol. 132, 529–543.

Nikaido, M., Kawakami, A., Sawada, A., Furutani-Seiki, M., Takeda, H., Araki, K., 2002. *Tbx24*, encoding a T-box protein, is mutated in the zebrafish somite-segmentation mutant *fused somites*. Nat. Genet. 31, 195–199.

Oates, A.C., Ho, R.K., 2002. *Hairy/E(spl)-related (Her)* genes are central components of the segmentation oscillator and display redundancy with the Delta/Notch signaling pathway in the formation of anterior segmental boundaries in the zebrafish. Development 129, 2929–2946.

Oates, A.C., Mueller, C., Ho, R.K., 2005. Cooperative function of *deltaC* and *her7* in anterior segment formation. Dev. Biol. 280, 133–149.

Parsons, M.J., Campos, I., Hirst, E.M., Stemple, D.L., 2002. Removal of dystroglycan causes severe muscular dystrophy in zebrafish embryos. Development 129, 3505–3512.

Pourquie, O., 2003a. The segmentation clock: converting embryonic time into spatial pattern. Science 301, 328–330.

Pourquie, O., 2003b. Vertebrate somitogenesis: a novel paradigm for animal segmentation? Int. J. Dev. Biol. 47, 597–603.

Roy, S., Wolff, C., Ingham, P.W., 2001. The *u-boot* mutation identifies a Hedgehog-regulated myogenic switch for fiber-type diversification in the zebrafish embryo. Genes Dev. 15, 1563–1576.

Topczewska, J.M., Topczewski, J., Shostak, A., Kume, T., Solnica-Krezel, L., Hogan, B.L., 2001. The winged helix transcription factor Foxc1a is essential for somitogenesis in zebrafish. Genes Dev. 15, 2483–2493.

van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Warga, R.M., Allende, M.L., Weinberg, E.S., Nusslein-Volhard, C., 1996. Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. Development 123, 153–164.

van Eeden, F.J., Holley, S.A., Haffter, P., Nusslein-Volhard, C., 1998. Zebrafish segmentation and pair-rule patterning. Dev. Genet. 23, 65–76.

Varga, Z.M., Amores, A., Lewis, K.E., Yan, Y.L., Postlethwait, J.H., Eisen, J.S., Westerfield, M., 2001. Zebrafish *smoothened* functions in ventral neural tube specification and axon tract formation. Development 128, 3497–3509.

Venters, S.J., Thorsteinsdottir, S., Duxson, M.J., 1999. Early development of the myotome in the mouse. Dev. Dyn. 216, 219–232.

Weinberg, E.S., Allende, M.L., Kelly, C.S., Abdelhamid, A., Murakami, T., Andermann, P., Doerre, O.G., Grunwald, D.J., Riggleman, B., 1996. Developmental regulation of zebrafish *MyoD* in wild-type, no tail and spadetail embryos. Development 122, 271–280.

Wolff, C., Roy, S., Ingham, P.W., 2003. Multiple muscle cell identities induced by distinct levels and timing of hedgehog activity in the zebrafish embryo. Curr. Biol. 13, 1169–1181.

Zhang, N., Gridley, T., 1998. Defects in somite formation in *lunatic fringe*-deficient mice. Nature 394, 374–377.